Identification of human milk α-lactalbumin as a cell growth inhibitor

Summary. A growth inhibitory protein, mammary inhibitory activity (MIA), was purified to apparent homogeneity from human milk. At concentrations of 5 to 10 ng/ml, the factor inhibited the growth of mammary epithelial cells by 30–80% and also inhibited the growth of normal rat kidney cells. Whereas the cell division of normal human mammary epithelium in primary culture was inhibited by MIA, cell division by fibroblasts from the same tissues was unresponsive. Inhibition was dose and time dependent and readily reversed when MIA was removed. MIA also inhibited growth in culture for three cell lines. The growth inhibitory protein migrated as a 14 kDa protein under reducing conditions on polyacrylamide gels in the presence of sodium dodecyl sulfate. The apparent isoelectric point was pI 5.0. The amino acid composition of MIA resembled that of α-lactalbumin, and sequence analysis of the N-terminal region comprising residues 1–24 and an isolated peptide were identical with the N-terminal and residues 66–81 of human α-lactalbumin. In addition, MIA was active in the lactose synthase system. The results strongly suggest that MIA and α-lactalbumin are identical proteins. Consistent with these results, α-lactalbumin preparations from several mammalian species, including human, goat, cow and camel, were all found to be growth inhibitory for cultured mammary epithelial cells. The inhibitory activity associated with human α-lactalbumin was destroyed by digestion with pepsin or chymotrypsin, by carboxymethylation of cysteine, or by cleavage of methionine 90 following cyanogen bromide treatment. The results raise the possibility that during lactation α-lactalbumin, a product of mammary cell differentiation, could be a physiologically relevant feed-back inhibitor of mammary cell growth and perhaps of other cell types as well.

Keywords: Milk protein; Mammary cells; Cell growth; Inhibition.

Abbreviations: MIA mammary inhibitory activity; MDGI mammary derived growth inhibitor; α-LA alpha lactalbumin; H-α-LA human α-lactalbumin; NRK normal rat kidney; IMEM improved minimal essential medium; DMEM Dulbecco's modified Eagles medium; FCS fetal calf serum; EGF epidermal growth factor; TGF β transforming growth factor β; CNBr cyanogen bromide; SDS sodium dodecyl sulfate; kDa kilodaltons; ND-PAGE non-denaturing polyacrylamide gel electrophoresis; TCA trichloroacetic acid.

Introduction

Human milk is known to be a source of factors that stimulate the growth of cells in culture. Numerous such growth stimulating factors have been purified, in some cases, to apparent homogeneity (Bano et al. 1985; Klagsbrun 1978, 1984; Salomon et al. 1984; Shing et al. 1984). Along with the growth factors, the importance of polypeptide growth inhibitors for the regulation of cell proliferation has already been established (Böhmer et al. 1985, Wang et al. 1986). Recently it was shown that lactating bovine mammary gland is the source of an effector called MDGI (mammary derived growth inhibitor), which prevents proliferation of cultured mammary epithelial cells in a dose-dependent, non-toxic and reversible manner (Böhmer et al. 1985, Herrmann et al. 1986, Kurtz et al. 1990).

In our studies, a growth inhibitor termed mammary inhibitory activity (MIA) was fractionated from human milk. Characterizations of MIA, including molecular weight, isoelectric point, amino acid analyses and par-

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tial amino acid sequence, revealed that MIA was similar to human α-lactalbumin (H-α-LA), the modifier protein of galactosyl transferase in the lactose synthase system.

Consequently, α-LA isolated from several mammalian species as well as chemically modified α-LA were compared with MIA and evaluated with respect to their affect on mammary cell growth in culture. The results reported herein suggest that α-LA is not only an end product of mammary cell differentiation and a regulator of lactose metabolism, but also may be a physiologically relevant growth inhibitor.

Material and methods

Preparation of milk inhibitor

The pH of human milk was adjusted to 4.6 with acetic acid to precipitate the caseins. The lipid layer was removed by centrifugation and the infranatant was dialyzed against 6 volumes of deionized water, and lyophilized. Following lyophilization, the protein was isoelectrically focused and isolated as previously described (Bano et al. 1985).

Fast protein liquid chromatography (FPLC)

Approximately 0.5–1 mg of protein from an IEF column (pI 5.0) was dissolved in 200 µl of Tris buffer 0.05 M, pH 7.4. The preparation was injected onto tandem columns of Superose-6 and -12 and chromatographed on a model LCC 500 FPLC system, Pharmacia. The proteins were eluted with Tris buffer (pH 7.4) containing 150 mM NaCl. One-half milliliter fractions were collected and aliquots evaluated for their affect on cell growth and protein synthesis using normal rat kidney (NRK) test cells. Multiple runs were performed and the peak fractions containing inhibitory activity were pooled and concentrated by lyophilization. The combined fractions were added to a Sephadex G-25 column, eluted with 0.1 M NH_4OH (pH 10), and concentrated using a spin vacuum apparatus. Following confirmation that inhibitory activity was recovered, a portion of the preparation was denatured in sodium dodecyl sulfate (SDS) and reduced with 2-mercaptoethanol for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

α-Lactalbumins

Several α-LA preparations from human, goat, bovine and camel were prepared according to published methods (Quarfoth and Jenness 1975).

Cell cultures

Human breast cancer cell line MCF-7 was maintained in improved minimal essential medium (IMEM) containing 10% fetal calf serum (FCS) (Richter et al. 1972). A1N4 cells (an immortalized normal human mammary epithelial cell line, Stampfer et al. 1980) were grown in DMEM (Dulbecco's modified Eagles medium) in 0.5% FCS in the presence of epidermal growth factor (10 ng/ml), insulin 10 µg/ml), transferrin (10 µg/ml) and hydrocortisone (0.5 µg/ml). NKR cells (clone F-49) supplied by Dr. J. DeLarco, National Cancer Institute, were grown on tissue culture dishes in DMEM supplemented with 10% FCS. Normal mouse and human mammary ducts and alveoli were isolated by collagenase digestion and filtration according to the procedure of Kidwell et al. (1984) and grown in IMEM medium, supplemented with 5% FCS.

Monolayer culture

Cells were seeded at a density of 1×10^5 cells/dish in 60 mm tissue culture dishes (Falcon, Lincoln Park, N.J.). Several concentrations of inhibitor were added. After 3 days of culture, the cells were trypsinized and counted in a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

For labeling experiments cell cultures were grown for 2–3 days in the presence of (L-4,5-^{3}H)-lysine (Amersham Corp., 83 Ci/mMol, 2 µCi/ml) with or without MIA, or native or modified α-LAs. All were added to the cultures at a concentration of 10 ng/ml. The growth medium was removed from the dishes and the cells were scraped into 5 ml of 0.5 M Tris-HCl, pH 7.4, 0.11 M NaCl, containing 1 mM unlabelled lysine. After centrifugation the pellets were re-suspended in 50 mM Tris-HCl, pH 7.6, 1 mM lysine and sonicated. Labelled protein was precipitated with 20% trichloroacetic acid (TCA) containing 1 mM lysine. The precipitates recovered by centrifugation were re-suspended in 5% TCA and re-centrifuged. The washing procedure was repeated three times. Precipitates were dissolved in 0.2 M NaOH and aliquots were directly counted in a beta counter.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) with appropriate buffer blanks and with bovine serum albumin or bovine α-LA as the standards.

S-Carboxymethylation

Approximately 300 mg α-LA was dissolved in 15 ml 6 M guanidine hydrochloride containing 0.18 M 2-mercaptoethanol and 0.1 M Tris-HCl buffer, pH 8.6. The solution was bubbled with nitrogen for 5 min and incubated at 37 °C for 12 h in a sealed container. Iodoacetic acid (2.7 mM), dissolved in 2 ml of 6 M guanidine hydrochloride containing 0.1 M Tris buffer, pH 8.6, was added to the solution with rapid stirring. The solution was allowed to stand for 5 min, then exhaustively dialyzed against water at 5 °C, lyophilized and stored at 5 °C (Brew et al. 1970).

Pepsin digestion

Fifty milligrams of α-LA were dissolved in 50 ml water and the pH adjusted to 2.0 with 0.1 N HCl. Pepsin (0.5 mg, Sigma) was added with stirring at 22 °C and the digestion allowed to proceed for 4 h. Samples were lyophilized and stored at 5 °C.

Chymotrypsin digestion

Fifty millligrams of α-LA were dissolved in 50 ml water and the pH adjusted to 8.0 with 0.1 N NaOH. Chymotrypsin (0.5 mg, Sigma) was added and the pH controlled at 8.0 using a Radiometer pH Stat by the addition of 0.1 N NaOH. The digestion proceeded for 4 h. Samples were lyophilized and stored at 5 °C.

Cyanogen bromide cleavage

An α-LA solution (14 mg/ml) in 70% formic acid was treated with 30 mg/ml CNBr at room temperature for 24 h. The protein was lyophilized, redissolved and lyophilized again to remove all unreacted CNBr (Doyen et al. 1979). Samples were relyophilized and stored at 5 °C.

Polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) and sodium dodecyl sulfate (SDS-PAGE) were run as described by Thompson and Brower (1988). Molecular weight standards for SDS-PAGE included α-LA (14 kDa), β-lactoglobulin (18 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa).

Amino acid analysis

Protein samples were hydrolyzed at 110 °C in a forced air oven for 24 h with 4.7 N HCl containing phenol (0.05%) in sealed, evacutated tubes. Analyses were performed in triplicate using a Beckman 119 CL amino acid analyzer. Cysteine was identified as 1/2 cystine by comparison of its retention time with a known standard. Data are reported as molar ratios with lysine fixed at 12 residues/molecule. Analyses performed by the above method have been described by Thompson et al. (1987, 1989) on bovine mammary and plant calmodulins, respectively.

Protein sequences analysis

The in situ digestion of proteins with protease was carried out as described by Bansal et al. (1989). Briefly, purified protein isolated from SDS-PAGE gels was digested in situ using endoproteinase Glu-c (EC 3.4.21.19, Boehringer-Mannheim) essentially as described by (Cleveland et al, 1977). The 15% separating PAGE gel was prepared according to Laemmli (1970) but the Tris concentration was made to 0.75 M instead of 0.375 M (Fling 1986). The stacking gels (5%) were prepared as usual but with large wells (26 mm × 3 mm × 1.5 mm). The gel slices were cut into small pieces and loaded into the well along with buffer containing 10% glycerol (v/v), 0.125 M Tris, pH 6.8, 6 mg (0.25%) bromophenol blue, and 0.1% SDS containing the enzyme. The protein to enzyme ratio was 50 : 1; to scavenge free radicals remaining in the gel 0.1 mM Na-thioglycolate was added to the running buffer in the upper chamber.

Electroblotting

Peptides were blotted onto a polyvinylidine difluoride membrane (Millipore Inc.) which were stained with Coomassie blue as described by Matsudaira (1978) and by Bansal et al. (1989).

Reversed phase HPCL

To approximately 0.05 mg each of MIA and H-α-LA was added a sufficient amount of starting solvent (70% water/30% acetonitrile with 0.1% trifluoroacetic acid) to give a final concentration of 10 μg/μl. Aliquots of 20 μl were injected onto a Bio-Rad C 4 reversed phase column (4.6 × 250 mm). The chromatogram was developed with a gradient of starting solvent and acetonitrile with 0.1% trifluoroacetic acid to 50% acetonitrile over 30 min at a flow rate of 1.5 ml/min; at 31 min the rate of addition of acetonitrile was changed to bring the acetonitrile to 75% by 40 min; the column was regenerated by a reverse gradient to starting solvent. Whey proteins were well separated by this procedure; the relative retention time for bovine serum albumin was 20.76 min.

Atomic absorption analysis

A Perkin Elmer Model 1100 atomic absorption analyzer unit was used for this study. Five milligrams of each α-LA sample was digested in 5% nitric acid for 16 h on a steam bath, after which they were analyzed.

ELISA for transforming growth factor (TGFβ)

$TGF\beta_1$ immunoreactivitiy was assessed in a double antibody ELISA as described by Lucas et al. (1990). This ELISA was specific for $TGF\beta_1$ in its active form. α-LA samples were dissolved in water at a concentration for 1 mg/ml. To activate any latent TGFβ which may have been present, samples (0.25 ml) were acidified to pH 2.0 by addition of 0.025 ml 0.12 N HCl and incubated ar 25 °C 30 min. Samples were neutralized by adding 0.018 ml of 0.14 N NaOH in 0.1 M HEPES, pH 7.2. Aliquots (0.1 ml) of untreated and acid-treated α-LA were diluted with 0.3 ml assay diluent (PBS containing 0.05% Tween 20, 0.5% BSA and 0.01% thimerosal) and 0.1 ml of that dilution was quantitated for $TGF\beta_1$ in the ELISA. Recombinant human $TGF\beta_1$ (Genentech Lot # 12704-9) was used as standard and as source of exogenously added TGFβ in some experiments. The assay range was 1–40 ng $TGF\beta_1$/ml; consequently, the sensitivity was 0.1 ng in the diluted samples. Intra- and inter-assay coefficients of variation were less than 10%.

Lactose synthetase

Lactose synthetase was assayed for stimulation by α-lactalbumin by the method of Palmiter (1969).

Results

The molecular weight of human milk-derived MIA purified by IEF, FPLC and gel filtration chromatography was determined on SDS-PAGE. As shown in Fig. 1, lane 1, a single, major protein band with a molecular weight of 14 kDa was detected for MIA. MIA (Fig. 1, lane 1) is slightly contaminated with light-chain IgG, MW_{app} 25 kDa, and serum albumin, MW_{app} 67 kDa, whereas H-α-LA (Fig. 1, lane 2) is somewhat more contaminated with both proteins.

A consistent pattern of inhibition of cell division as measured by the Coulter Counter was observed when MIA was tested on different primary cell cultures (Fig. 2). Normal human mammary epithelium from reduction mammoplasty was inhibited by 70% at 10 ng MIA/mL. In contrast fibroblasts from human mammary tissue were unresponsive to MIA. Normal mouse mammary epithelium from primiparous animals was also sensitive to MIA, with 40% reduction of cell division observed at 10 ng MIA/ml (Fig. 2). In addition, the inhibition of normal mouse mammary epithelial cells increased progressively as a function of time (Fig. 3).

In the case of cultured cells, immortalized human (A 1N 4) and neoplastic (MCF-7) mammary cell lines exhibited an inhibition of cell growth of about 80 and 40%, respectively, at an MIA concentration of 10 ng/ml of media (Fig. 4).

Incorporation of [^{3}H] lysine into total cell protein by NRK cells was 80% inhibited in the presence of 10 ng/ml of MIA as shown in Fig. 5. Inhibition of NKR cell

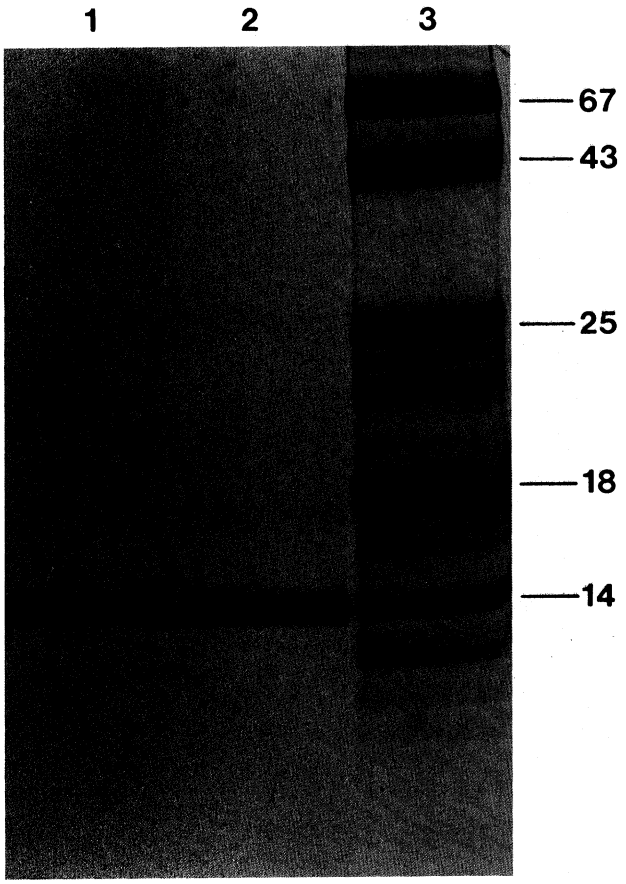

Fig. 1. SDS-PAGE of isoelectrically focused milk fraction MIA (*1*), H-α-LA (*2*), and molecular weight standards (*3*). The proteins were stained with Coomassie blue

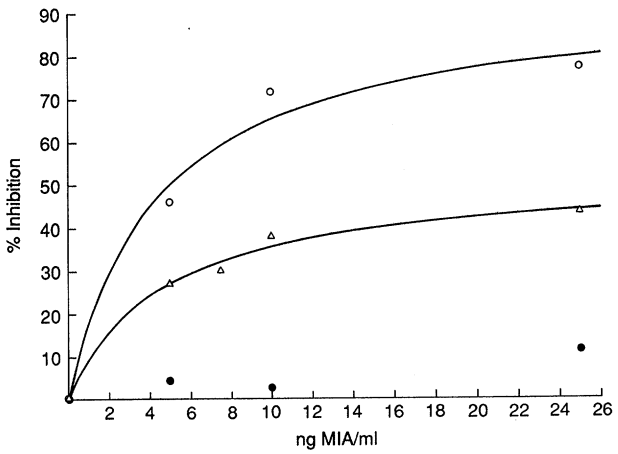

Fig. 2. Dose dependent effect of MIA on cell division of normal human (O-O) and mouse (Δ-Δ) mammary cells and on human mammary fibroblasts (●-●). Cells were grown on tissue culture plastic dishes and incubated with MIA at the indicated concentrations for 72 h. The experiment was repeated 3 times. Solid lines represent fit of data with Eq. 1 to one experiment. Table 1 gives the average fit of the data

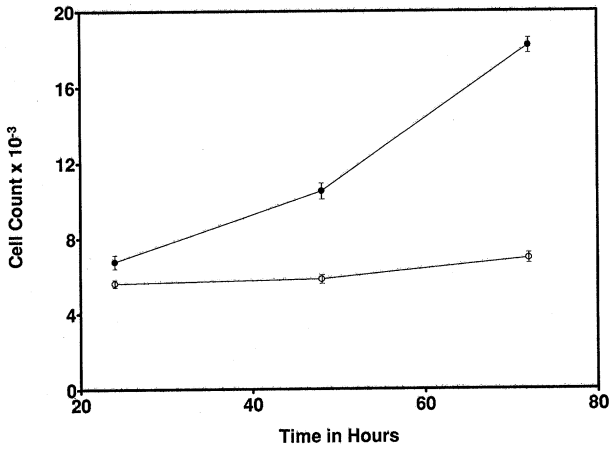

Fig. 3. Time dependent inhibition of cell division by MIA. Growth curves are shown in the presence of (O) and absence (●) of MIA. Normal mouse mammary epithelial cells were cultured on plastic dishes in the presence of 10 ng/ml of MIA and processed at different time intervals. Error bars represent standard deviation. Number of replicates: 3

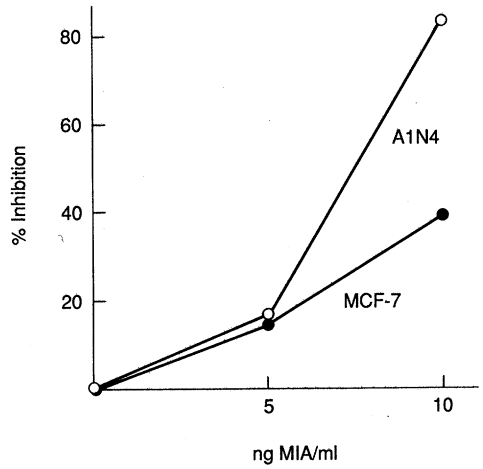

Fig. 4. Effect of MIA on immmortalized (O-O) and neoplastic (●-●) human mammary cell growth. Cells were grown in culture conditions as described in Materials and methods. MIA was added at 5 and 10 ng/ml concentrations and cell counts were done after 72 h using a Coulter Counter. Number of replicates: 3

division by MIA also showed a dose response similar to that for lysine incorporation into protein in these cells (Fig. 5).

The curves shown in Figs. 2 and 5 all have the apparent shape of a saturation binding isotherm. Using nonlinear regression analyses these curves were fitted directly with Eq. 1:

$$RGI = \frac{I_{max} \cdot C}{K_I + C} \quad (1)$$

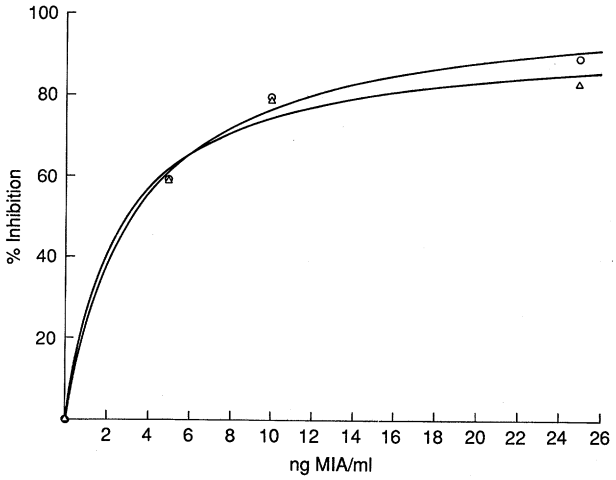

Fig. 5. Dose dependent effect of MIA on uptake of [^{3}H]lysine (O-O) by NRK cells. The incorporation of labeled lysine into total cell protein in NRK cell layers was estimated after treating the cells with MIA at different concentrations at 72 h. MIA effect on NRK cell division (Δ-Δ). NRK cells were incubated with MIA at the indicated concentrations and the affect on cell growth was analyzed at 72 h. The experiment was repeated 3 times. Solid lines represent fit of data with Eq.1 for one experiment. Table 1 gives the average fit of the data

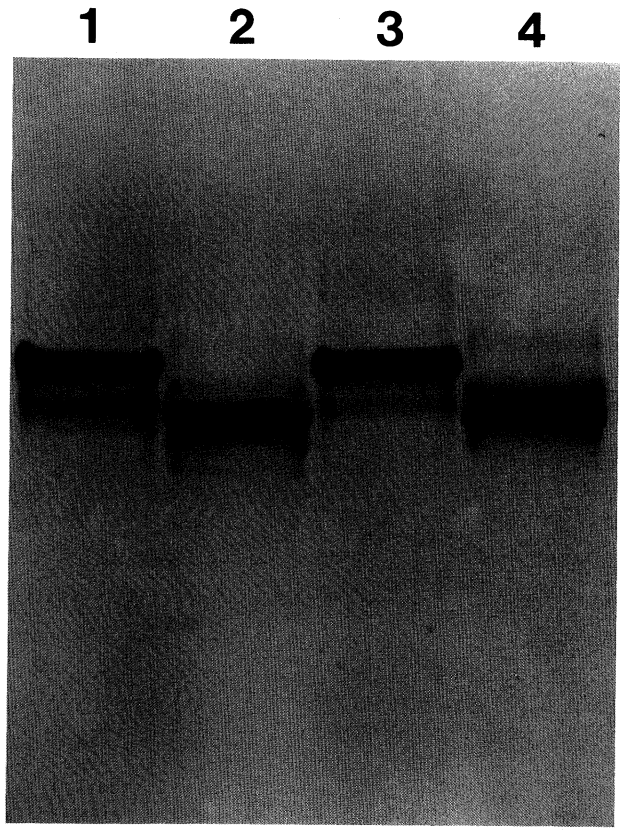

Fig. 6. ND-PAGE of H-α-LA (*1* and *2*) and MIA (*3* and *4*). 10 mM [ethylene bis (oxyethylenenitrilo)] tetraacetic acid was added to the samples in *2* and *4*, and 10 mM $CaCl_2$ to *1* and *3*

where RGI is the relative percent of growth inhibition (^{3}H incorporation or cell division) which is dependent upon the concentration (C) of the effector. Analysis yields I_{max}, or the maximum inhibition of the system and K_I, or the concentration at half maximum inhibition. This analysis can be considered to be an extension of the theory of thermodynamic linkage in which the changes in a property of a system are linked in a concentration dependent way to binding of a component of the system. This type of analysis has been used successfully in the study of a number of biological sytems (Farrell et al. 1988, 1990). In a similar fashion, the study of hormone receptors has been facilitated by use of a mathematically transformed version of Eq. 1, the Scatchard plot. Results of these analyses are summarized in Table 1 which gives the maximum percent inhibition and K_I for each tissue or cell type. The I_{max} values vary with cell type, but the K_I values for MIA are similar and range from 2.6 to 4.5 ng/ml (Table 1). MIA inhibitory affects on normal mouse mammary epithelial cells, plated on Type I and Type IV collagen

Table 1. Analysis of affects of MIA dose dependent effects on cells

Cell type	Function measured	K_I(ng/ml)	I_{max} (% maximum inhibition)
Human mammary epithelia[a]	cell division	4.32 ± 1.77	93.2 ± 10.9
Mouse mammary epithelia[a]	cell division	4.54 ± 1.08	51.8 ± 3.8
Human mammary fibroblasts[a]	cell division	–[b]	10.0 ± 1.0
NRK cells[c] (clone F 49)	[^{3}H]lysine incorporation	3.37 ± 0.64	101 ± 5
NRK cells[c] (clone F 49)	cell division	2.60 ± 0.82	93.3 ± 6.2

[a] Analysis of data shown in Fig. 2
[b] Effect too small to be fitted (Fig. 2)
[c] Analysis of data shown in Fig. 5

substratum, were tested. Inhibition was somewhat greater when the cells were plated on Type IV collagen than on Type I collagen, though in the range observed for cells grown on uncoated plates.

Gel electrophoresis of MIA in the presence of SDS showed it to have a mobility similar to that of α-LA (Fig. 1). Although both preparations contain slight impurities, they appear to be identical in molecular weight (14 kDa) with reference to standards. This similarity prompted a comparison of the chemistry of the two proteins. Thompson and Brower (1988) and Thompson et al. (1989) have demonstrated that α-LA undergoes a calcium dependent electrophoretic shift on ND-PAGE. Figure 6 demonstrates that MIA and H-α-LA not only migrate the same distance on ND-PAGE but each undergoes the same electrophoretic shift, $R_m = 1.13$, which confirms that MIA, like H-α-LA, is a calcium binding protein. The Ca^{2+} dependent shift for α-LA is not observed by SDS-PAGE as it is with calmodulin (Thompson et al. 1988).

Reversed phase HPLC showed that the major component of MIA had a retention time greater than that of bovine α-LA (26 vs 32 min) but identical with the retention times for two H-α-LA preparations. MIA and these two H-α-LA preparations were subjected to amino acid analyses. The results of these 24 h hydrolyses are given in Table 2 and compared with the H-α-LA sequence-based composition. The values are nearly identical, with the exception of the lowered contents of Cys and Met in MIA. However, the exact contents of these two amino acids are difficult to determine with small amounts of protein and a single hydrolysis time. Despite these differences, application of the equation $S\Delta n$ (Cornish-Bowden 1983), shows a strong correlation ($S\Delta n = 28$) of MIA to H-α-LA, equal to the correlation between MIA and baboon α-LA ($S\Delta n = 27$). On this scale a value of 52 or less is an indication of strong compositional relatedness.

To confirm the identity of MIA and H-α-LA, sequence analyses were carried out. The MIA was first electrophoresed on SDS-PAGE and digested in situ with endoproteinase Glu-c as described in Materials and methods. Two major peptides were blotted from the electrophoretogram of this digest and sequenced. The results are given in Table 3. The sequence of Peptide-1 was identical to the N-terminal 24 residues of H-α-LA including the Cys at position 6. Analyses of Peptide-2 showed it to be identical with residues 66 to 81 of H-α-LA including Cys 80. Combined with the above PAGE, HPLC, amino acid, and molecular weight anal-

Table 2. Amino acid analyses of mammary inhibitory protein as compared with human α-lactalbumin

Amino acid	Human α-LA		MIA	Human α-LA sequence
	prep. #1	prep. #2		
Lys	11.60 (12)[a]	11.84 (12)	10.76 (11)	12
His	2.26 (2)	2.30 (2)	3.47 (3)	2
Arg	1.12 (1)	1.02 (1)	1.41 (1)	1
Asp	14.66 (15)	14.80 (15)	12.59 (13)	16
Thr	6.39 (6)	6.37 (6)	6.16 (6)	7
Ser	6.75 (7)	6.87 (7)	7.58 (8)	8
Glu	15.50 (15)	15.47 (15)	15.04 (15)	15
Pro	2.80 (3)	2.52 (2)	2.89 (3)	2
Gly	6.04 (6)	6.08 (6)	6.90 (7)	6
Ala	5.39 (5)	5.28 (5)	5.56 (5–6)	5
$^1/_2$ Cys	7.89 (8)	6.23 (6)	2.93 (3)	8
Val	2.65 (3)	1.82 (2)	3.76 (4)	2
Met	1.86 (2)	1.65 (2)	0.75 (1)	2
Ile	11.00 (11)	11.09 (11)	9.61 (10)	12
Leu	14.27 (14)	14.34 (14)	12.70 (13)	14
Tyr	4.19 (4)	4.66 (5)	3.82 (4)	4
Phe	4.00 (4)	4.00 (4)	4.00 (4)	4
Trp	n.d.	n.d.	n.d.	4

[a] Rounded off number is in parenthesis

n.d. Not done

Table 3. Comparison of amino acid sequence of MIA and human α-lactalbumin peptides[a]

	1 5 10 15 20
MIA, peptide-1	K Q F T K *C* E L S Q L L K D I D G Y G G I A L P
H-α-LA, N-terminal	K Q F T K *C* E L S Q L L K D I D G Y G G I A L P
	66 70 75 80
MIA, peptide-2	S T E Y G L F Q I S N K L W *C* K
H-α-LA, residues 66–81	S T E Y G L F Q I S N K L W *C* K

[a] Sequence analysis of the peptides was done according to the published procedures as described in Materials and methods

Table 4. Affect of α-lactalbumins on protein synthesis in NKR cells[a]

Factor	Concentration (ng/ml)	Treatment	% Inhibition
Human MIA	10	–	69
Human α-LA	10	–	53
Human α-LA	10	pepsin	2
Human α-LA	10	chymotrypsin	0
Human α-LA	10	CNBr	11
Bovine α-LA	10	carboxymethylated	0
Bovine α-LA	10	–	69
Goat α-LA	10	–	50
Camel α-LA	10	–	50

[a] NRK cells were incubated with different α-LA preparations at a concentration of 10 ng/ml and the affect on protein synthesis was determined as described in the legend of Fig. 5. Other proteins tested under these conditions at 10 ng/ml were: β-lactoglobulin, lactoferrin and α_{s1}-casein from bovine milk, as well as bovine γ-globulin and hen egg white lysozyme; none of these proteins had any significant inhibition in this assay

yses, it appears that the major protein component of MIA is H-α-LA.

The effect of purified α-LA from different species on lysine incorporation into protein by NKR cells is shown in Table 4. At 10 ng/ml MIA and bovine α-LA, both inhibited this incorporation (protein synthesis) by 70%. Inhibition was 50% with camel, goat and H-α-LA. However, after CNBr treatments, or pepsin or chymotrypsin digestions, H-α-LA was no longer inhibitory. Other bovine milk proteins tested under these conditions were without effect (Table 4).

No significant levels of heavy metal contamination (less than ten parts per billion) were observed in MIA, α-LA, or modified α-LA preparations, confirming that inhibition of cell growth and protein synthesis was most likely not due to heavy metal ions. Lyophilized salts from the purification buffers do not produce the observed inhibition either, so that the most likely conclusion is that these growth inhibitory effects are due solely to protein. One possible potent growth inhibitory protein is TGFβ (Roberts et al. 1985). The $TGF\beta_1$, content of the α-LA preparations from human and bovine sources, used in the inhibitory studies, was determined by ELISA assay as described in Materials and methods. None of these samples contained detectable levels of of $TGF\beta_1$. The level of detectability in these assays is 1.0 ng/ml and the amounts of α-LA used were 250 μg/ml. A sample of H-α-LA, prepared from colostrum (early milk) and not used in the growth inhibitory assays was also analyzed. This latter sample contained 4.4 ng $TGF\beta_1$/mg α-LA; or a 0.0004% contamination. Recovery of exogenously added $TGF\beta_1$ to α-LA samples averaged 83% suggesting only a slight interference in the assay.

Lastly to confirm that MIA and H-α-LA are highly similar if not identical, MIA was analyzed for its ability to modify bovine galactosyl transferase (Palmiter 1969) in the lactose synthase system. MIA substitutes as the

modifier although exhibiting approximately 50% of the activity of bovine α-LA per mg in the assay with rat mammary microsomes as the source of the enzyme (Leung et al. 1989).

Discussion

We have purified a protein from human milk which we termed MIA, mammary inhibitory activity. At low concentration (10 ng/ml) the protein inhibited growth of human mammary epithelial cells by up to 80%; however, fibroblasts from the same tissues were unresponsive (Fig. 2). Growth inhibition by MIA was dose and time dependent and was readily reversed when MIA was removed. MIA inhibited lysine uptake of NKR cells as well as inhibiting cell division (Fig. 4). Remarkably, all of the assays yielded K_Is in the range of 2.6 to 4.5 ng/ml, as disclosed by analysis of the inhibition curves. Human α-LA substituted for MIA in the NRK lysine incorporation experiment and produced similar inhibition at identical concentrations (Table 4). In fact α-LA from a variety of species substituted for MIA in these experiments. Chemical modifications of H-α-LA, including methionine cleavage with CNBr, carboxymethylation of cysteine, or pepsin/chymotrypsin hydrolysis, decreased its ability to inhibit cell growth and protein synthesis in culture. Glycosylated bovine α-LAs (results not shown) also inhibited protein synthesis but by 30% less than their non-glycosylated counterparts. Since α-LA has been demonstrated to bind a variety of heavy metal ions (Murakami et al. 1982, Musci et al. 1985), the possibility that heavy metal contamination in the MIA and α-LA preparations was responsible for the inhibitory activity was tested by atomic absorption analyses. Although traces of Mn^{2+}, Pb^{2+}, Hg^{2+}, Ni^{2+}, and Zn^{2+} were detected, the concentration were less than 10 parts per billion, concentrations that are far below those capable of inhibition of cell growth. From Table 1 it can be calculated that the overall K_I for NRK cells is 2.98 ng/ml; this corresponds to a 210 pM concentration of α-LA. Roberts et al. (1985) demonstrated that TGFβ is a bifunctional regulator of cell growth and can both stimulate and inhibit NRK cells. The effective dose at which half their growth is inhibited (ED_{50}) was found to be 10 to 30 pM (Roberts et al. 1985). Thus if MIA and α-LA samples were contaminated at up to a 10% level, TGFβ could be the actual inhibitory agent. TGFβ is active in mammary tissue during growth and development of the gland (Akers 1990) but its activity decreases as full lactation begins; if it were present in mammary gland then it could possibly contaminate milk proteins. ELISA analyses of the human and bovine α-LAs used in the inhibitory study showed no $TGF\beta_1$. An α-LA (human) prepared from colostrum (early milk) did contain 0.0004% $TGF\beta_1$. Thus even under conditions where TGFβ would be expected to be greatest in mammary tissue, the purified α-LA does not contain a sufficient amount of $TGF\beta_1$ to cause inhibition of cell growth. We have therefore ruled out salts, heavy metals and one potent growth inhibitor, as possible contaminants. While it is possible that some other factor could be the true growth inhibitor, it would have to be tightly bound to α-LA, be present in milk, and copurify across a wide variety of species (bovine, goat, camel, and human; Table 4).

It was not unexpected that enzymatic hydrolysis (pepsin, chymotrypsin) or S-carboxymethylation of H-α-LA would destroy its ability to inhibit cell proliferation and protein synthesis.

Carboxymethylation of cysteine negates the abilitiy of α-LA to bind Ca^{2+} (Thompson et al. 1988) by unfolding the molecule. It was not anticipated, however, that CNBr modification of the single methionine (residue 90, located within the calcium binding elbow; Stuart et al. 1986) would destroy most of the biological activity of the molecule toward cell cultures. CNBr-treated bovine α-LA (also one methionine at position 90) participates as the modifier protein of galactosyl transferase, although the K_m for glucose is increased several fold whereas there is no change in the velocity of the reaction (Berliner et al. 1991). The argument we advance is that α-LA, as a Ca^{2+} modulated protein, requires the integrity of the Ca^{2+} binding elbow (Stuart et al. 1986) to fully potentiate biological processes. Variations in the ability of α-LAs isolated from different species to affect protein synthesis are shown in Table 4. Unlike calmodulins, the primary amino acid sequences of α-LAs across genera are not highly conserved.

The similarity of MIA with H-α-LA seems unequivocal from the data presented in this study. First, H-α-LA and other α-LAs substitute for MIA in cell growth and protein synthesis inhibition assays. Second, the amino acid analyses (with the exception of cysteine) in conjunction with $S\Delta n$ calculations, support the proposal that the proteins are closely related or identical. Third, the sequence for two peptides of MIA show 100% identity with regions of H-α-LA. Fourth, MIA and H-α-LA migrate exactly the same on ND-PAGE and both undergo the Ca^{2+} dependent electrophoretic shift with R_m values of 1.13. Further, on reducing SDS-PAGE both proteins migrate with a MW_{app} of 14 kDa. Fifth,

by reversed phase HPLC, MIA and H-α-LA elute at identical retention times of 32 min. This elution time is significantly greater than for bovine α-LA which contains fewer hydrophobic amino acids. Sixth, MIA, like H-α-LA from other species, modifies galactosyl transferase in lactose synthase albeit at a reduced level. Based upon these criteria, we conclude that MIA and H-α-LA are functionally and structurally identical proteins.

Although fast protein liquid chromatography was used to purify MIA, it is apparent that much larger quantities of the identical protein, H-α-LA, can easily be prepared using standard methods (Quarfoth and Jenness 1975) and if necessary, the human protein crystallized as described by Fenna (1982) or baboon α-LA as reported by Aschaffenburg et al. (1979). Approximately 0.4% of the weight of human milk is H-α-LA. Though further experimentation will be needed to elucidate the mechanism of action of H-α-LA (or α-LA of other species) in the inhibition of mammary epithelial cell growth, the results presented here raise the possibility that α-LA is a physiologically relevant inhibitor of mammary and NRK cell proliferation. It is tempting to speculate that mammary cell proliferation and the ultimate cessation of cell growth as differentiation occurs, is a consequence of the inhibitory action of α-LA, a natural product of mammary cell differentiation (autocrince regulation). It is also tempting to speculate that α-LA could modulate growth of other tissues as well, especially during lactation. It is well known that serum proteins "leak" into milk by a paracellular route despite tight junctions in mammary epithelia. However, the reverse process occurs: the presence of α-LA in the sera of lactating cows and women has been documented by radioimmune assays (Akers et al. 1986, Simickova et al. 1987). The serum concentrations of α-LA in lactating cows and women averaged 196 ng/ml and 204 ng/ml respectively, nearly 50 times the average value calculated for K_I in this study (Table 1). In non-lactating, non-pregnant, healthy women the α-LA in serum averages less than 1 ng/ml. It could be that the leakage of α-LA into serum modulates cell growth in non-mammary tissues, thus maintaining the known diversion of metabolic energy necessary for milk synthesis.

Although MIA and α-LA share several properties with another inhibitor, MDGI factor, found in bovine milk by Böhmer et al. (1985), sequence analyses of MIA and α-LA clearly distinguish them from MDGI. However, Humphreys-Beher et al. (1987) reported that α-LA was able to modulate the growth response of parotid acinar cells "positively and negatively". While acknowledging that the mechanism(s) of action of α-LA on cell growth was not understood, they pointed out "that α-LA will chelate into lipid bilayers depending on pH, thereby affecting membrane fluidity and protein mobility. Many membrane bound enzymes, including glycosyltransferases, are sensitive to changes in membrane fluidity. Thus the bimodal response of α-LA may be related not only to steric interactions with cell surface 4-β-galactosyltransferase but also to alterations in enzyme activity as a consequence of lipid membrane interactions of the protein as well." DeGeyter et al. (1989) have shown that bovine α-LA has the ability to decapacitate mouse sperm inhibiting binding of the sperm to zona pellucida. Most recently, Farrell and Thompson (1990) have demonstrated that both β-lactoglobulin and α-lactalbumin can inhibit the activity of mammary derived acid phosphatases which are secreted into milk. This inhibition is mediated through protein-protein interactions which can be reversed by Ca^{2+} binding to weakly ($K_D \sim 3$ mM) interacting sites, thus indicating a possible role for these proteins in regulating phosphate content of milk.

Reminiscent of other Ca^{2+} modulated proteins (i.e., calmodulin) is the observed multifunctional nature of α-LA, including regulation of cell proliferation, phosphate regulation (Farrell and Thompson 1990), and its well-known modification of galactosyl transferase for the synthesis of lactose (demonstrated by the classical of Ebner et al. 1966). It would not be surprising, therefore, if other functions of this unique Ca^{2+} binding protein are reported.

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